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Bioproduction of azadirachtin and its biological activity against *Spodoptera littoralis*

Maher I. Aly, Mohamed N. Barakat*, Asmaa S. Elsayed and Ahmed F. El-Aswad

Pesticide Chemistry and Technology Dept., Faculty of Agriculture, Alexandria University

**Crop Science Dept., Faculty of Agriculture, Alexandria University.*

ABSTRACT

Many plant products possess properties that give the same effect of pesticides and are known to be used in pest management strategy. Currently, attention is being focused on the use of neem-based botanical insecticide. Azadirachtin is highly interesting compound for both its complex chemical structure and its synthesis and for its biological properties. Callus produced from leaves of neem tree, *Azadirachta indica* A. Juss, produced in vitro bionatural insecticide azadirachtin when grown in modifies MS medium. The optimum light condition for callus incubation and propagation was 2,000 lux, 16h. Azadirachtin isolated by standard procedure depend on solvent partition program. The crude extract amount was higher in methanol fraction (F_3) compared to that in petroleum ether (F_1) and methylene chloride fraction (F_2) using TLC and UV-scanning and HPLC techniques for detection and determination of azadirachtin. It is interesting to observe that the methylene chloride fraction (F_2) contained the highest concentration of azadirachtin than the others (F_1 0.245, F_2 13.467 and F_3 0.108 g/100g callus). The general growth inhibition percentages of azadirachtin on *Spodoptera littoralis* Boisd larvae in different fractions arranged between 61.8-57.6%. The required to kill 50% of the larvae (LT_{50}) was 1.87 days for azadirachtin methylene chloride extraction, 1.99 days for petroleum ether of azadirachtin fraction and 2.17 for methanol fraction of azadirachtin, while neemazal takes 4.10 days to kill 50% of the tested larvae. The antifeedant percentage of azadirachtin in different fractions at 0.5 mg kg^{-1} was studied using no-choice bioassay method. Antifeeding activity percentage of azadirachtin were 18.8% for petroleum ether fraction, 49.3% for methanol fraction and 59.4% for methylene chloride fraction while neemazal (5%) antifeeding activity percentage was 87.1%. The results of this study show that azadirachtin derived from neem leaves callus by tissue culture needs an applied approach to produce the compound in a commercial production.

Key words: *Azadirachta indica*, *Spodoptera littoralis*, tissue culture, callus induction, botanical insecticides, antifeedant.

INTRODUCTION

In recent years, much effort has been payed towards the development of natural insecticides originating from plants. In this context, currently, attention is being focused on the use of neem-based botanical insecticide (Wei-hong and Ban-qian, 2006). Over 300 compounds have been isolated from various parts of the neem tree among them the terpenoids that comprise. The major active compounds commonly refused to as limonoids. Azadirachtin, the chief substance and the best known example of these limonoids is accumulated in the seed kernels of *Azadirachta indica* (Sujanya *et al*., 2008). The compound and its analogues are potent insect antifeedants and growth regulators (El-Aswad *et al*., 2003; Abdelgaleil and El-Aswad, 2005; Charleston *et al*., 2005; Abou-Tarboush *et al*., 2009). Insects from different orders differ markedly in their responses to azadirachtin. Insects from Lepidoptera are the most sensitive to these compounds as effective antifeedants, concentration ranged from 1-50 ppm and some times less depending upon species (Martinez and van Emden, 2001). Coleoptera, Hemiptera and Homoptera were less sensitive (Mordue and Nisbet, 2000).

Much interest has been paid to understand the complex structure and synthesis of azadirachtin and its biological properties as will as biosynthesis (Morgan, 2009). In order to obtain information about these aspects of azadirachtin, we have employed tissue culture techniques for its production (Wewetzer, 1998). Future approaches may include such method to produce azadirachtin for research as will as commercial aspects and insect control. Cell culture technology has been used to produce valuable compounds that lack a synthetic or chemical synthesis procedure (Sujanya *et al*., 2008). Plant cell and tissue culture systems are complementary and may provide competitive metabolite production systems when compared to whole plant extraction. Several types of high-yielding tissue culture systems have been developed. These include the cultivation of specific organ cultures, suspension cultured cells selected for high productivity on production media and high-density culture (Lui and Staba, 1981; Zieg *et al*., 1983). Azadirachtin production of *A. indica* tissue and cell suspension culture using Murashige and Skoog (MS) medium supplemented with different nutrients and plant growth regulators was studied by (DiCosmo and Misawa, 1995; Babu *et al*., 2004; Kalafalla *et al*., 2007; Sujanya *et al*., 2008). It was showed that the production of azadirachtin in quantites depends on the components of the nutrient medium and growth regulator type and concentration.

The aim of this work is to investigate in vitro bio-production of azadirachtin and to evaluate its biological activity on the cotton leafworm, *Spodoptera littoralis*. The mortality percentage, larval weight gain and consumed diet were monitored. Also, the growth inhibition and antifeedant activity percentages were calculated.

MATERIALS AND METHODS

Tested compounds: Azadirachtin (95%), it is obtained from Sigma Co., It was used a reference in HPLC analysis. Neemazal (5% EC), it was obtained from Agriculture Research Center, Cairo, Egypt. It was used a standard compound in the biological activity experiments.

Neem plant: The neem tree, *Azadirachta indica* A. Juss., a member of the Meliaceae family produces the insecticidal liminoid, azadirachtin (Kraus, 2002). Leaves were used to source of explant. The neem seedlings obtained from the Agricultural Research Center, Giza, Egypt.

Tested insect: A susceptible cotton leafworm, *Spodoptera littoralis* (Boisd.), strain was obtained from a stock culture maintained under constant condition of 28 ± 1ºC and 65 ± 5% R.H. The culture started with egg-mass introduced from the Plant Protection Research Center, Dokki, Giza Gavernorate.

Preparation of MS medium and modified MS medium: The MS medium was prepared according to Murashige and Skoog (1962). To prepare MS-m medium which used for callus induction, one liter of the MS medium was supplemented with plant growth regulators, 1 mg of 6-Benzyl amino purine (BAP), 0.8 mg of Kinetin (KN) and 6 mg of Adenine sulphate (Eeswara *et al*., 1998). Agar was added after adjusting the pH to 5.8 to obtain solidified media. Also, to obtain MSp-1 medium which used in cell suspension culture, one liter of MS medium without agar, 2 mg of 1-Naphthaleneacetic acid (NAA) and 0.5 mg of BAP were mixed (Dixon, 1985). In addition, micropropagation medium (MS-g medium) was prepared using one liter of solidified MS medium, 0.1 mg of BAP, 0.1 mg of KN and 0.6 mg Adenine sulphate.

Preparation and inoculation of explants: The equipments and media were autoclaved at 121 ºC for 30 min. (for media) or 60 min. (for equipments) under pressure of 15 PSI. The new leaves of the neem seedlings were washed and cleaned carefully with running water. The leaf explants were allowed to soak in 75% ethanol solution for one minute followed by two times rinsing with sterile distilled water. Then, 15 minutes in 0.1% mercuric chloride and washed with six changes of sterile distilled water. Under laminar flow hood, the sterile leaves were cut into small pieces using scalpel and the leaf pieces were placed adaxial side down into Petri dishes containing 25 ml of agar-solidified culture medium (MS-m). The five explants of the same seedling were placed in one Petri dish. The culture dishes were then incubated in the culture room at 20 ºC and under dark condition or 2,000 lux, 16 hours using fluorescent lamps.

Callus and cell production: After six weeks incubation, callus induction was recorded. The calli derived from explants could be divided into two groups; a group was used to initiate the cell suspension culture. Another group of calli was transferred to MS-g medium at 20 ºC and 2,000 lux, 16 hours using fluorescent lamps. After other six weeks, the produced greater calli were care yielded and kept to extract of azadirachtin from them. Also, cell suspension culture was initiated by placing pieces of a friable tissue culture. This friable callus transferred to MSp-1 moving liquid medium. The cell suspensions were maintained at 150 rpm on a rotary shaker at 16/8 h photoperiod. After filtration, filtrates at 5 weeks were obtained.

Extraction of produced azadirachtin: The extraction of azadirachtin from callus was carried out according to the method described by Allan *et al*. (1994). Accumulated freeze-dried callus obtained from tissue culture media, known weight was homogenized with methanol in glass homogenizer and filtered. The callus residue was resuspended in methanol and the filtration repeated twice. The solvent was removed on a rotary evaporator, keeping the temperature below 40 ºC, and the residue was redissolved in methanol + water (66 + 33 by volume), and this solution was partitioned against light petroleum distillate (b.p. 60-80 ^{o}C). The organic phase was removed (Petroleum ether extract, Fraction 1) and the aqueous methanol partitioned against dichloromethane to obtain dichloromethane extract (Fraction 2). The remaining aqueous methanol sample gave (Fraction 3). All extracts (Fraction 1, 2 and 3) were evaporated to dryness and kept to analysis. Also, the produced azadirachtin was extracted from cells and filtrates according to the extraction protocol described by Sujanya *et al*. (2008). The filtrates of cell suspension containing the extracellular components were extracted into methanol of high performance liquid chromatography (HPLC) grade using the same procedure of the extraction from callus. The fractions were utilized for HPLC analysis.

Analysis of azadirachtin: The qualitative analysis was carried out on silica plates pre-coated on aluminium foil (Fluka Chemie, Switzerland) (Mandava, 1985). The different extracts were eluted with methylene chloride + acetone (1 : 1) solvent system, allowed to dry, then exposured to UV-lamp. In addition, the production of azadirachtin in the culture media was confirmed by UV-scanning of the extracts using Visible/UV-Spectrophotometer, Nicolet, Thermo.

The quantitative analysis of azadirachtin in different fractions using HPLC technique was carried out according to Allan *et al*. (1994) with slight modification. IBM-Compatible PC with Gynkot-Software was connected with a high performance liquid chromatography (HPLC) system consisting of modifier pump (ConstaMetric 3000), gradient programmer (GM-4000), high pressure mixer, pulse dampener (Negretti, Southampotn, UK), variable wavelength UV detector (SpectroMonitor 3100) set at 217 nm and an oven containing a model 7125 loop injector fitted with a 20 µl loop (Rheodyne, Cotati, USA), backpressure regulator (Tescom, Elk River, USA) and the analytical column.

Antifeeding and growth-inhibition of azadirachtin: The extracted azadirachtin (in dryness crude extract of different solvents) of callus and cell suspension were evaluated for its antifeedant and growth inhibitory effects on the second instar larvae of *Spodoptera littoralis* using no-choice bioassay. The extracts were mixed with the artificial diet (Bakry *et al*., 1973) to give concentration of azadirachtin (0.5 mg kg^{-1}). Control diet was prepared with a maximum volume of each solvent (petroleum ether, methylene chloride, methanol, acetone) alone used in the treated diet. After evaporation of solvent, 1 g of treated diet and 10 pre-weighed second-instar larvae were placed in each Petri dish (9 cm). Three replicates of each extract and each concentration were carried. After 10 days of feeding on treated diet, the survival larvae of the three replicates of each concentration were collected in one plastic box and fed on the same treated diet until 21 day. The diet consumed by each larva was determined daily by weighing the remaining diet of each treatment. The percentage antifeedant index was calculated from the following equation: % antifeedant = 100 (C - T/C), were C is the weight of diet consumed in control and T is the weight of diet consumed in the treatment. Also, larval growth inhibition was assayed relative to control based on larval weight gain. The growth inhibition was calculated from this equation: Growth inhibition = $[(C_L - T_L)/C_L] \times 100$, where C_L is the larval weight gained in the control and T_L is the larval weight gained in the treatment. In addition, number of died larvae was daily recorded. LT_{50} values were calculated according to Finny (1971). Moreover, T-test was performed by using of Software, Costat (CoStat Statistical Software, 1990).

RESULTS AND DISCUSSION

Response of explant to callus induction: Data of the production of callus derived from leaf explants of neem, *Azadirachta indica* were recorded after six weeks incubation. These explants were incubated on MS-m medium protocol. The percentage of explants that developed calli indicated that the highest of callus induction (100%) while, the lowest value (32%) were obtained from the incubation under light (2,000 lux, 16h) and dark condition, respectively (Table 1). Also, it was showed that the growth rate of the callus was dependant on the incubation conditions. The weight of callus derived from explant under light condition was significantly higher than that of explant under dark condition.

Table 1: Percentage and quantity of callus induction for *Azadirachta indica* under different conditions

	Light condition	Dark condition
Callus induction (%)[1]	100	32
Callus yield (g/100 explant)[2]	20	3.76

[1] Values are highly significant ($P = 7.251 \times 10^{-5}$ ***),
[2] Values are significant ($P = 0.047$*) based on the T-test.

The statistical analysis indicated that the induction and weight of callus were significantly influenced by light/dark conditions of the incubation. Therefore, the light condition is the optimum condition for incubation to callus induction and propagation. In general, before utilizing tissue culture technique as a tool in chemical bioproduction, it is necessary to determine and optimize the factors influencing callus formation, its quality and quantity during induction and maintenance. The previous results provide an indication of the relative important of incubation conditions on culture response and growth regulator type and concentration (Gautam *et al*., 1993; and Gharyal *et al*., 1983).

Qualitative and quantitative analysis of azadirachtin:

Extraction and qualitative analysis: Freeze dried callus was submitted to a standard procedure for the isolation of azadirachtin (Johnson *et al*., 1994). The extract was partitioned between aqueous methanol and light petroleum distillate to give a non-polar petroleum extract (referred to as F1) while the aqueous methanol partition was submitted to a second solvent partition against dichloromethane. Thus, dichloromethane extract (F2) and the aqueous methanol residue (F3) were obtained. The qualitative analysis of different extracts (F1, F2, F3), and extract of azadirachtin 25% was carried out by TLC analysis using (methylen chloride : acetone, 1:1) solvent system. The bands were detected by UV spectrum. On major band was observed in all tested extracts. Also, with UV-scanning, it was observed the UV absorbance in all extracts was at 217 nm, this value identical with that obtained by Allan *et al*. (1994); Babu *et al*. (2006); Sujanya *et al*. (2008).

Quantitative analysis: The different extracts were evaporated to dryness by rotary evaporator, some samples required to left under room condition about 3 days. The quantity of the crude extracts was determined. (Table 2) indicated that the quantity of crude extract of callus and cell suspension filtrate (extracellular components) is higher in F3 fraction than that in F1 or F2 fraction. The highest amount of crude extract in F3 or the total of fractions was obtained from callus. Azadirachtin concentration in different fractions was determined by HPLC using UV-detector. Azadirachtin (95%) in methanol was used a reference. The results of HPLC analysis indicated that the R_t of azadirachtin = 4.2 min. Azadirachtin amounts (g/100g dryness crude extract) in different fractions of callus and cell suspension filtrate are presented in (Table 3). The highest concentration of azadirachtin was detected in methylene chloride (F2) for callus. No promising concentration of azadirachtin was detected in all fractions of cell suspension filtrate.

Table 2: Weight of air dried crude extracts in different fractions extracted from callus of tissue culture and filtrate of cell suspension (g/100g source)

	Callus	Filtrate
Petroleum ether fraction	0.065	0.060
Methylene chloride fraction	0.235	0.013
Methanol fraction	2.225	0.320
Total	2.525	0.393

Table 3: Concentration of azadirachtin in air dried crude extracts in different fractions extracted from callus and filtrate (g /100g dried crude extract)

	Callus	Filtrate
Petroleum ether fraction	0.245	1.245
Methylene chloride fraction	13.467	0.915
Methanol fraction	0.108	0.985
Total	13.820	3.145

In general, the results indicated that the crude extract amount was higher in methanol fractions (F3) compared to that in petroleum ether fraction (F1) and methylene chloride fraction (F2). Although, the concentration of azadirachtin compound was higher in F2 than that in F1 and F3. The greater amount of crude extract in F3 and containing lower azadirachtin quantity were expected because it contained a large amount of polar components. It was expected also the slight amount of crude extract and azadirachtin concentration in F1 because it contained a large amount of non-polar components. It was interested observed that the fraction F2 contained large concentration of azadirachtin. These results are agreement with results obtained by Allan *et al*. (1994) who indicated that the analysis chromatogram of methylene chloride fraction (F2) showed a larger peak for azadirachtin compared to that in F1 and F3.

Biological activity: The larvicidal effect of the extracted azadirachtin (0.5 mg kg^{-1} artificial medium) against the larvae of cotton leafworm, *S. littoralis* was evaluated using no-choice bioassay method. Values of the time required to kill 50% (LT_{50}) expressed as days and 95% confidence limits are shown in (Table 4).

Table 4: LT_{50} values of azadirachtin in different fractions of callus and neemazal

	Azadirachtin conc. (ppm)	LT_{50} and Confidence limits
Petroleum ether fraction	0.5	1.99 (2.31 – 1.70)
Methylene chloride fraction	0.5	1.87 (2.42 – 1.41)
Methanol fraction	0.5	2.17 (2.71 – 1.71)
Neemazal (5%)	10	2.01 (2.34 – 1.70)
	5	4.10 (4.67 – 3.60)

In this experiment until the terminal (21 days), the mortality percent did not exceeded 10% of different solvents and 3.33% of control.

The lowest value of LT_{50} was 1.87 days caused by azadirachtin in methylene chloride fraction. The azadirachtin of neemazal even 5 and 10 mg kg^{-1} has LT_{50} of 4.10 and 2.01 days, respectively. Almost the same LT_{50} values of azadirachtin were recorded in methanol fraction, 0.5 mg kg^{-1} and neemazal, 10 mg kg^{-1} (Table 4).

During feeding for 21 days on treated diet with azadirachtin in different fractions and in neemazal. The growth inhibition percentage of azadirachtin (Table 5) showed that the highest effect of all fractions was detected at 3rd week. Neemazal treatments caused growth inhibition of 100% at 2nd week.

Table 5: Growth inhibition percentage of azadirachtin in different fractions of callus and neemazal

	Azadirachtin conc. (ppm)	1st week	2nd week	3rd week	General
Petroleum ether fraction	0.5	0.3	33.7	225.0	57.6
Methylene chloride fraction	0.5	12.9	37.1	277.3	61.8
Methanol fraction	0.5	65.6	31.6	133.8	61.6
Neemazal (5%)	10	85.8	100.4	---	99.4
	5	74.9	99.5	---	99.3

The general growth inhibition percentages of azadirachtin in different fractions arranged between 61.8-57.6%. The antifeedant percentage of azadirachtin in different fractions at 0.5 mg kg^{-1} and in neemazal extract 5 and 10 mg kg^{-1} is shown in (Table 6).

Table 6: Antifeeding activity percentage of azadirachtin in different fractions of callus and neemazal

	Azadirachtin conc. (ppm)	1st week	2nd week	3rd week	General
Petroleum ether fraction	0.5	-12.3	15.2	42.8	18.8
Methylene chloride fraction	0.5	53.1	52.8	71.1	59.4
Methanol fraction	0.5	43.3	41.9	61.7	49.3
Neemazal (5%)	10	38.4	74.0	---	87.1
	5	-14.5	64.2	---	80.4

Azadirachtin in methylene chloride fraction showed the highest antifeedant activity through 3 weeks with general of antifeedant percentage of 59.4 %. It was observed that all tested larvae died after two weeks in the two treatments of neemazal. However, the general of antifeedant percentage of 80.4 and 87.1% for concentrations of azadirachtin in neemazal, 5 and 10 mg kg^{-1}.

The results of the biological activity experiment indicated that azadirachtin extracted in F2 has high effectiveness compared to that in F1 and F3. In similar, Allan *et al*. (1994) found that F2 which contains azadirachtin was significantly and completely deterred from feeding. The use of neem as a pesticide is well known (Ascher, 1993) and extracts obtained from seed, bark and leaf of the neem tree have been successfully investigated as environmentally acceptable bioinsecticides for use in crop protection (Mordue and Blackwell, 1993). All these extracts were obtained from intact neem plant organ. Here in this study we used known concentration of azadirachtin in extract obtained from in vitro production. Therefore, the higher biological activity against cotton leafworm recorded might be attributed to the higher purified quantity of azadirachtin and its molecule found in the active configuration in the in vitro induced callus compared to that of the intact leaf. Charleston *et al*. (2005) and Abou-Tarboush *et al*. (2009) stated that azadirachtin and its analogues are potent insect antifeedants. It is well known that in vitro cultures are able to produce secondary metabolites, sometimes even in quantities that allow economically feasible production (Fujita *et al*., 1981). Furthermore, previous studies (Zhao *et al*., 2001) have reported that callus culture could provide an alternative supply of secondary metabolite for use in medicine, stimulating the production or inducing the biosynthesis of novel compounds not found in vivo. This is because in callus cultures cells are undifferentiated, which means the genes that are in control of secondary metabolite production may be turned off or not under specific control (Wink, 1986).

In conclusion, the results of this study shows that azadirachtin in extracts derived from neem callus have demonstrable larvicidal activities, therefore, further work is needed to produce azadirachtin in a large scale using neem callus system reactor for the commercial production.

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الانتاج الحيوى للأزدراختين وتأثيره البيولوجى ضد دودة ورق القطن

ماهر ابراهيم على – محمد نجيب بركات*- أسماء السيد- أحمد فرحات الاسود
قسم كيمياء وتقنية مبيدات الأفات- كلية الزراعة- جامعة الإسكندرية
*قسم المحاصيل- كلية الزراعة- جامعة الإسكندرية

العديد من المنتجات النباتية لها نفس خصائص تأثير المبيدات الحشرية وبذلك يمكن استخدامها في استراتيجية ادارة مكافحة الآفات. لذا يزداد الاهتمام حاليا لاستخدام المركبات المنتجة من النيم خاصة الأزدراختين. الكالس الناتج من أوراق شجرة النيم والمنزرع على بيئة مناسبة أظهر قدرته على انتاج المبيد الحشرى الطبيعى أزدراختين والذى تم استخلاصه باتباع طريقة قياسية للتوزيع مع المذيبات متدرجة القطبية . وقد تم اجراء تعرف وصفى للمركب بطريقة الفصل الكروماتجرافى بالطبقة الرقيقة وكذلك باجراء مسح بالأشعة فوق البنفسجية وتقديره كميا باستخدام HPLC. وأيضا تم تقدير النشاط البيولوجي على دودة القطن وذلك بتغذية اليرقات على بيئة صناعية معاملة بتركيز (0.5 مجم/كجم). وأظهرت النتائج أن الظروف المثلى لتحضين بيئة تكوين وانتاج الكالس هى الضوء . وكانت كمية المستخلص المجفف هوائيا فى مستخلص الميثانول أعلى من مستخلص البتروليم ايثر والمثلين كلوريد بينما تركيز الأزدراختين كان أعلى فى مستخلص المثلين كلوريد . التركيز المختبر من الأزدراختين والمحضر من مستخلص المثلين كلوريد كان أعلى تأثيرا على يرقات دودة ورق القطن من حيث الزمن اللازم لموت 50% حيث كان (1.87 يوم) ومن حيث النشاط كمانع للتغذية وقد كان متوسط التأثير خلال ثلاث أسابيع من التغذي 59.4%.